ZOOLOGICAL SCIENCE 10: 489-496 (1993)

Binding of Thyroid Hormone to Freshwater Perch Leydig Cell Nuclei-rich Preparation and its Functional Relevance

Nihar R. Jana and Samir Bhattacharya[1]

Department of Zoology, Visva-Bharati University, Santiniketan-731 235, W. Bengal, India

ABSTRACT—Leydig cells were isolated from the testis of a freshwater perch, *Anabas testudineus*, belonging to the prespawning stage of reproductive cycle. Cells were sonicated and a pure nuclei preparation was obtained for binding assay. Under optimum assay conditions of pH 7.4 at 30°C and 90 min incubation binding of [^{125}I] 3,5,3′-triiodothyronine (T_3) to Leydig cell nuclei was saturable at 1.7 nmol/l concentration. A Scatchard analysis of T_3-binding exhibited a Kd of 26.8×10^{-9} mol/l and a maximum binding capacity (B_{max}) as 1.66 pmol/mg DNA. Competitive inhibition studies demonstrated that binding of T_3 to Leydig cell nuclei is analogue specific. Biological relevance of T_3 binding to perch Leydig cell was evaluated by adding varied concentrations of T_3 to Leydig cell incubation (1×10^6 cells/incubation). Twenty five ng to 100 ng of T_3 resulted in a dose dependent increase in androgen release while 200 ng of T_3 had no additional effect over 100 ng under present incubation system. Stimulation of androgen release by T_3 was significantly inhibited ($p<0.01$) by cycloheximide. T_3 (100 ng/ml) increased protein synthesis in Leydig cell (70% as compared to control) which was significantly inhibited ($p<0.01$) by cycloheximide. Results indicate that binding of T_3 to Leydig cell of perch testis triggers the synthesis of a protein (or proteins) which in turn stimulates androgen release.

INTRODUCTION

Thyroid has long been implicated in the reproduction of fish [11, 13–16, 20, 26, 29]. Annual cycles of thyroid activity in teleostean fish have been found to be correlated with the gonadal maturation [6, 8, 17]. Experimental hypothyroidism in teleosts effected retardation of ovarian development [23] and administration of thyroid hormone resulted in the maturation of oocytes in stellate sturgeon [10], goldfish [16] and freshwater perch [30]. All these reports clearly indicate an influence of thyroid hormone on teleostean gonadal function, but how it does so is still unclear.

Recent reports from this laboratory demonstrated high affinity and low capacity triiodothyronine (T_3) binding sites in the ovarian nuclei of perch [7, 22]. These information indicate a direct involvement of thyroid hormone in piscine ovarian function. However, report regarding the influence of thyroid hormone on male reproductive function in fish is lacking. The aim of this paper is to show T_3 binding to Leydig cell nuclei isolated from the testis of a freshwater perch, *Anabas testudineus* and also to demonstrate that the addition of T_3 to Leydig cell incubation *in vitro* causes significant increase in androgen release.

Accepted April 17, 1993
Received August 19, 1992
[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Aniaml

Anabas testudineus is a freshwater perch predominantly found in Eastern India. It breeds during the monsoon and its reproductive cycle can be divided into four phases [6]. Perch belonging to prespawning stage were used for the present experiment as thyroid hormone level in plasma remains high during this phase [6]. Adult male perch (25–30 g) length (10–12 cm) were acclimatized in laboratory aquaria at 28°–30°C for at least 7 days prior to experiment. They were fed *ad libitum* with commarcial fish food (Shalimar Fish Food, Bird and Fish food Manufacturers, Bombay, India).

Testis weight varied from 200–300 mg.

Preparation of Leydig cells from perch testis

The testis of the perch is encapsulated by tunica albuginea, a thin delicate membrane which envelopes the fibrous connective tissue. The connective tissue fiber from the peripheral stroma divides the lumen of testis into a large number of seminiferous tubules of varied shape and size. Each tubule contains germ cell at different developmental stages and Sertoli cell. Germ cell can be categorised as primary and secondary spermatogonia, spermatocyte, spermatid, and spermatozoa. Perch testis has discrete interstitial cell or Leydig cell between the seminiferous tubules. In histological section of perch testis stained with haematoxylin and eosin, interstitial or Leydig cells appear as a large cell with oval nuclei. Plenty of Leydig cells from perch testis could be observed under microscope. This is in contrast to other freshwater teleosts like the Indian catfish, *M. vittatus* and murrel, *C. gachua* where testis are devoid of true interstitium and characterised by possession of tubule boundary cells. In these fishes it is difficult to identify the Leydig cell. Since this perch, *Anabas testudineus* contains clearly defined Leydig cell in considerable number, the testes were collected by killing the perch, sliced with a surgical blade and then subjected to the preparation of Leydig cell according to the method described by Yu *et al.* [36] and Yu and Wang [37] which is a modification of the procedure reported by Dufau *et al.* [12] and Van Damme *et al.* [32]. Briefly small pieces of testis from perch were transferred to Erlenmyer flask containing preincubation medium (MEM with 25 mM HEPES, 0.1% BSA, 0.1% sodium bicarbonate, pH 7.4; penicillin 1000 U/ml; streptomycin 50 μg/ml). The testis pieces were gently dispersed for 10 min with a magnetic stirrer placed on ice bath and a completely homogeneous sunpension was obtained. The cell suspension was then filtered through a fine nylon mesh and preincubated for one hr at 30°C with shaking at 50 cycles/min. This was then cooled under ice and centrifuged at 400 g for 10 min at 4°C. Pelleted Leydig cells were suspended in the incubation medium which was a preincubation medium with 0.125 mM xanthine plus sodium heparin, 0.5% (v/v). It appears that testicular Leydig cells of perch differs from that of mammalian Leydig cells as the latter could be harvested by centrifugation at 250 g [37].

Purity of Leydig cells

To examine the purity of the Leydig cell preparation, histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme was performed by a modification of the method of Wiebe [35]. The reaction mixture contained 0.1 M Na-K phosphate buffer, pH 7.2, 0.14 μmol 5α-androstan 3β-ol-17-one, 0.6 μmol nitroblue tetrazolium, 7 μmol NAD. 500 μl of reaction mixture was added to 1×10^6 cells and the suspension was incubated for one hr at 30°C. We have described earlier that 30°C was the optimum temperature for incubating the tissues from this fish [7, 8, 29]. 5α-A-3β-ol-17-one was omitted from control incubation. A drop of the cell suspension was placed on a glass slide and allowed to dry. After embedding in gelatin, positively stained cells was estimated by counting a minimum of six field under the light microscope. Approximately more than 85% cells were found to be 3β-hydroxysteroid dehydrogenase positive indicating them to be Leydig cells.

Binding incubation

T_3 binding to nuclear preparations from Leydig cells was performed by following the description reported earlier from this laboratory [7]. Leydig cells were lysed by ultrasonication (Labsonic 2000, B. Braun, West Germany) at 150 KHz and nuclei were isolated according to our earlier procedure [7, 22] adopted from the description of Lawson *et al.* [19]. To determine the optimum binding conditions of [^{125}I]-T_3 to Leydig cell nuclear preparations, incubations were performed at different temperatures pHs and time intervals. It was found that 90 min of incubation at pH 7.4 and temperature 30°C were optimum conditions of radiolabelled T_3 binding (data not shown). 30 μg of DNA was incubated in a final volume of 500 μl with varying concentrations of [^{125}I]-T_3 (0.26–2.2 nmol/l) in the absence (total binding) or presence of 500 fold excess of unlabelled T_3 (nonspecific binding) at pH 7.4 and 30°C for 90 min in a shaking water bath. After termination of incubations, free

and bound radioactivity were separated by the addition of ice cold 40% polyethylene glycol (PEG-mol. wt. 6000) and by centrifugation at 3000 g in a refrigerated centrifuge. The supernatant was aspirated and radioactivity of the pellet was measured in a Gamma Counter (1282 Compugamma CS, LKB, Sweden).

Incubation of Leydig cells

For *in vitro* incubation, Leydig cells were suspended in MEM (300 μl, containing 1×10^6 cells) and was added to each well of multiwell module (NUNC, Denmark). Incubations were performed at 30°C with gentle shaking under an atmosphere of oxygen. Cells were allowed to incubate for 2 hr and at 2 hr hormone and other chemicals were added, incubation was then continued for another 3 hr. Hence total incubation period was 5 hr. A 2 hr preincubation time was necessary for the recovery of cells. Viability of cells were determined at the beginning and at the end of incubation by Trypan blue (0.1%) dye exclusion method which showed 90–80% viability of the cells. For each experiment, functional ability of isolated Leydig cells in *in vitro* incubation was checked by the addition of purified carp gonadotropin [2], which released androgen in the medium. T_3 was added in increasing concentrations (25–200 ng/ml) to the Leydig cell incubation. T_3 was dissolved by the addition of 10 μl of NaOH (1N) to 1 ml of T_3 solution (distilled water). 10 μl volume was fixed for each concentration of T_3 which was added to 300 μl of Leydig cell incubation. Same concentration and volume of NaOH was added to control incubation (without T_3) as vehicle. Since MEM contained 25 mM HEPES, there was no changes of pH due to this addition. After termination of incubation, each incubate was centrifuged at 500 g, medium was collected and stored at −20°C until androgen RIA.

Protein synthesis in Leydig cells

To monitor the protein syunthesis in Leydig cells in response to T_3, cells (1×10^6/well) were incubated with [^{14}C]-leucine (specific activity −300 mCi/mmole) in MEM supplemented with 1 mM of eighteen different amino acids except leucine. The pH was adjusted to 7.4. Protein synthesis of Leydig cells were determined according to the procedure previously described from this laboratory [7]. Briefly, cells were incubated in the presence of T_3 (100 ng/ml) alone or T_3 (100 ng/ml) plus cycloheximide (50 μg/ml) or in the absence of either chemicals (control). The cells were then separated from the medium by centrifugation, resuspended in distilled water and subjected to ultrasonication at 150 KHz. Amount of protein in the lysed preparation was determined by following the method of Lowry *et al.* [21] taking bovine serum albumin as the standard. The sonicated material was precipitated with a final concentration of 10% TCA. The pellet obtained after centrifugation at 3000 g in a refrigerated centrifuge was first washed with 10% TCA followed by another wash with 5% TCA containing cold leucine (1 mM). 7% TCA was then added to the pellet and the sample was heated for 30 min at 95°C to denature nucleic acids. After centrifugation at 3000 g and two subsequent washes with ethanol : ether (1 : 1), the final precipitate was dissolved in 500 μl of 1(N) NaOH and counted in a Liquid Scintillation Counter (LSS 20, ECIL) in 10 ml of toluene based cocktail containing PPO, POPOP and methyl cellosolve and TCA precipitable radioactivity is expressed as dpm/mg protein.

Radioimmunoassay of androgen

RIA of androgen was carried out according to the procedure described by Yu and Wang [37] which was a modification of a method reported earlier [1]. The medium collected after Leydig cell incubation was subjected to androgen RIA. It was found that androgen values were similar with or without extraction by diethylether. The antiserum used (provided by Dr. John Y. L. Yu, Academia Sinica, Taiwan, batch no. ASIZ-T-3-02) had 100% crossraction with testosterone, 74% with 5α-dihydrotestosterone, 1.23% androstenedione, 0.59% androstenediol and very negligible crossreactions with other steroids. Sample, [^{3}H]-testosterone and antitestosterone serum were added and the samples were incubated for 18 hr at 4°C. Dextran coated charcoal was used to separate bound from free steroid. After centrifugation at 3000 g, supernatant containing the bound hormone was counted in a Liquid Scientillation Counter. The sensitivity

of the androgen RIA was 5.0 pg and the linear range of standard curve was 5–300 pg. The interassay co-efficient of variation was less than 10%.

Statistics

Data were analysed by one way analyses of variance (ANOVA). Where F value indicated significance means were compared by post hoc multiple range test. All values are expressed as mean ± standard error of the mean (SEM).

RESULTS

T_3 binding to perch Leydig cell nuclei

Nuclear preparation from Leydig cells were incubated at 30°C for 90 min at pH 7.4 and T_3 binding was one half maximal by 45 min and maximal at 90 min under these conditions. To obtain the specific binding of T_3, 30 μg of DNA were incubated with increasing concentrations of [^{125}I]-T_3 (from 0.26 nmol/l to 2.2 nmol/l). Figure 1 shows that specific binding of T_3 increased linearly from 0.26 nmol/l to 1.7 nmol/l and then reached a plateau indicating saturation of binding sites. Specific binding data were analysed by Scatchard plot which showed a Kd of 26.8×10^{-9} mol/l and maximum binding capacity (B_{max}) was 1.66 pmol/mg DNA (Fig. 2). Competitive binding experiments were conducted at optimal assay conditions with variation in T_3, TRIAC (3,3′,5-triiodothyroacetic acid), T_4 and carp GtH (cGtH-Banerjee *et al.* [2]) concentrations. Results clearly indicate that T_3 binding sites in Leydig cell nuclei are analogue specific (Fig. 3). T_3 inhibited the binding of [^{125}I]-T_3 very efficiently but less competitively than TRIAC while T_4 was a poor competitor. cGtH did not inhibit [^{125}I]-T_3 binding. Tissue specificity of T_3 binding was observed by incubating nuclear preparations of testis, liver, tail kidney and small intestine. Maximum specific binding occurred with liver nuclei followed by testis. Binding was negligible with small intestine and tail kidney nuclei (Fig. 4).

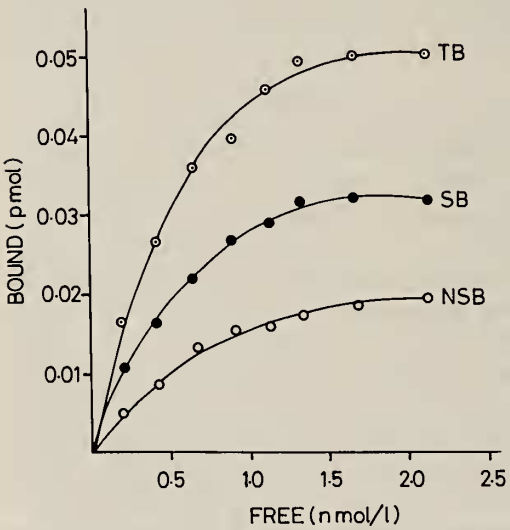

Fig. 1. Saturation curve of T_3 binding to Leydig cell nuclei. 30 μg of DNA from the Leydig cell nuclei were incubated with 0.26–2.2 nmol/l of [^{125}I]-T_3 for 60 min at 30°C. Non-specific binding (NSB) is in the presence of 500 fold excess cold T_3. Specific binding (SB) is the difference between total binding (TB) and non-specific binding. Data represents the mean ± SEM of six determinations.

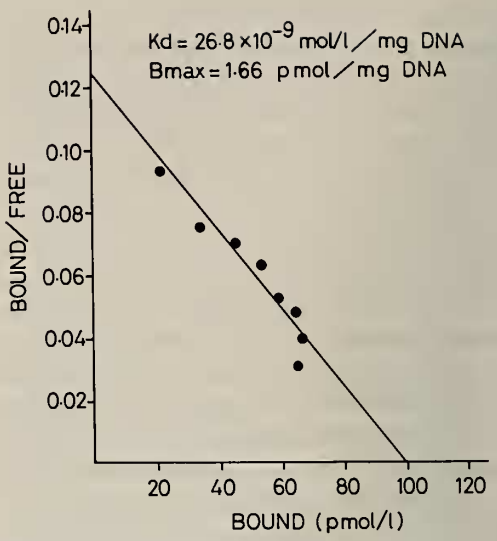

Fig. 2. Scatchard analysis of the data obtained from Fig. 1. The X axis represents p mol/l concentration of bound T_3 and Y axis represents bound to free ratio. Kd: Dissociation constant. B_{max}: Maximum binding capacity.

Effect of T_3 on androgen release from Leydig cells

Addition of increasing concentrations of T_3 to

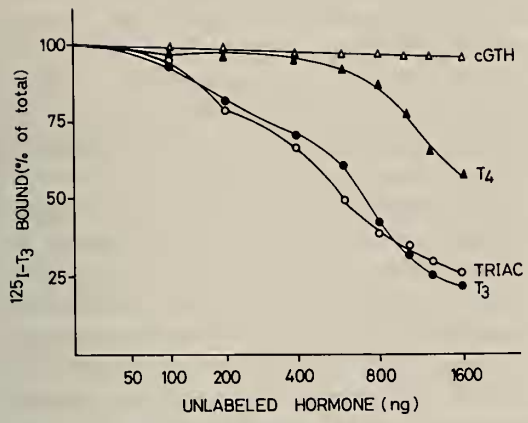

FIG. 3. Competitive inhibition of [^{125}I]-T_3 binding to perch Leydig cell nuclei by TRIAC, T_3, T_4, and cGTH. All values are mean ± SEM of six determinations.

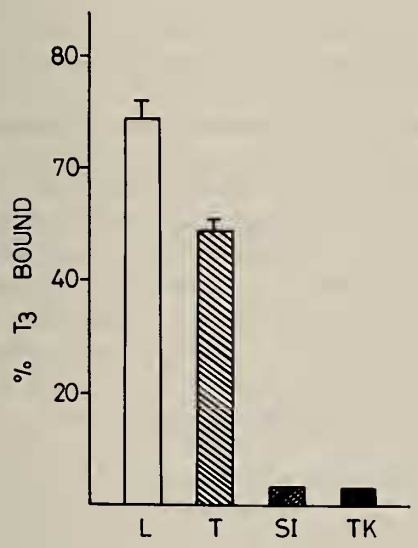

FIG. 4. [^{125}I]-T_3 binding to nuclei obtained from different tissues. Liver (L), Testis (T), Small intestine (SI), Tail kidney (TK). All values are mean ± SEM of six determinations.

Leydig cell incubation resulted in dose dependent increase of androgen release. Increase of androgen release was observed from 25 ng to 100 ng, further increase in dose had no additional effect in the present incubation system (Fig. 5).

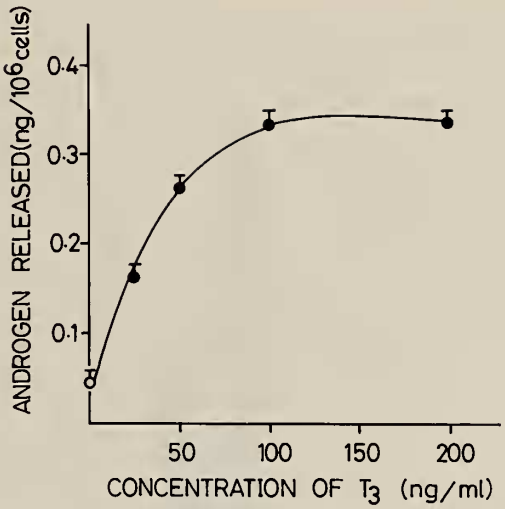

FIG. 5. Effect of T_3 on androgen release by Leydig cell of perch. After a preincubation of 2 hr the cells were incubated for another 3 hr in the absence of T_3 (control) or in the presence (increasing concentrations) of T_3. Data represents the mean ± SEM of six determinations.

TABLE 1. Inhibition of T_3-stimulated androgen release by cycloheximide

System	Androgen released (pg/1×10^6 cells)
Control	49.9 ±6
T_3 (100 ng/ml)	345* ±9.8
T_3 (100 ng/ml) + Cycloheximide (50 μg/ml)	40** ±2.1

P*<0.01 as compared to control.
P**<0.01 as compared to T_3 added experiment.
Values are mean ± SEM of six determinations.

This stimulation of androgen release by T_3 was inhibited by cycloheximide (Table 1).

Stimulation of Leydig cell protein synthesis by T_3

Incubation of Leydig cell with [^{14}C]-leucine and T_3 resulted in a significant ($p<0.01$) increase in TCA precipitable radioactivity which was cycloheximide sensitive (Fig. 6). T_3 caused about 70% increase in protein synthesis as compared to control while cycloheximide inhibited this stimulation to about 53%.

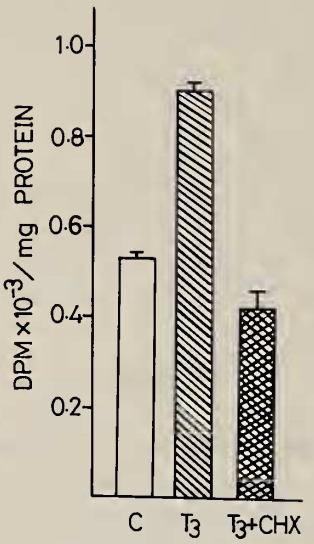

FIG. 6. Protein synthesis in the Leydig cell in reponse to T_3. Incubation of [^{14}C]-leucine into TCA precipitable protein was determined in presence (100 ng/ml) or in the absence of (control) of T_3 or T_3 (100 ng/ml) with 50 μg/ml cycloheximide (CHX). Data represents the mean ± SEM of six determinations.

DISCUSSION

The present study demonstrates that nuclear T_3 binding sites are present in Leydig cell of perch testis. Binding of T_3 to teleostean Leydig cell nuclei is a new report and suggests an influence of thyroid hormone on testicular function. This freshwater perch breeds once a year *i.e.* during monsoon or rainy season and its annual reproductive cycle consists of four stages [6]. We have selected prespawning stage male fish because gonadal metabolic activity is in peak during this stage. Standardisation of T_3 binding assay showed maximum binding at 30°C with pH 7.4. These two characteristics clearly coincided with the natural environment of prespawning stage (April–May) when water temperature varied between 28°–32°C and pH of the water is weakly alkaline (7.3–7.5). This indicates that optimal binding conditions may have physiological relevance.

Liver is the conventional binding site for thyroid hormone and commonly used for thyroid hormone receptor assay in teleost. The Scatchard plot analysis of our data shows that T_3 binding affinity to perch Leydig cell nuclei is comparable to hepatic nuclei of the same fish. $Ka = 0.041 \times 10^9\ M^{-1}$ in perch Leydig cell nuclei and $Ka = 0.06 \times 10^9\ M^{-1}$ in the hepatic nuclei of the same fish [7]. These values are closer to the affinity of hepatic nuclei of rainbow trout ($Ka = 0.22 \times 10^9\ M^{-1}$) [33] while hepatic nuclei of coho salmon has much higher affinity ($Ka = 1.03 \times 10^9\ M^{-1}$) [9]. Surprisingly T_3 binding affinity of perch ovarian nuclei is much higher ($Ka = 0.11 \times 10^9\ M^{-1}$) as compared to Leydig cell nuclei [7]. Occupation of receptor site by T_3 shows considerable variation. T_3 binding capacity was considerably higher in perch ovarian nuclei ($B_{max} = 4.312$ pmol/mg DNA) [7] as compared to Leydig cell nuclei ($B_{max} = 1.66$ pmol/mg DNA). Hepatic nuclei of perch showed five fold greater T_3 binding capacity ($B_{max} = 8.882$ pmol/mg DNA) [7] in comparison to Leydig cell nuclei. However T_3 binding to Leydig cell nuclei of perch is analogue and tissue specific.

Specific binding of T_3 is believed to result in the initiation of at least some of the effect of thyroid hormone [24]. Oppenheimer *et al.* [25] detected low concentration of nuclear T_3 binding sites within rat brain, spleen and testis, these tissues are supposed to be unresponsive to thyroid hormone. Unresponsiveness has been defined as a failure of thyroid hormone in increasing O_2 consumption. But in testis with relatively low thyroid hormone binding sites a small change in O_2 consumption induced by thyroid hormone may not be detectable [28]. Recently T_3 binding sites have been detected in rat testis Sertoli cell nuclei and a role of thyroid hormone in the regulation of growth and maturation of Sertoli cell has been suggested [18]. A novel C-erb-A gene encodes a protein which is a receptor for thyroid hormone and this gene has also been isolated from human testis library [3, 27, 34]. The binding of T_3 to perch Leydig cell nuclei is therefore not entirely unexpected but it obviously raises a question whether perch testis is functionally responsive to thyroid hormone or not.

To answer this question perch Leydig cells were incubated *in vitro*. Addition of T_3 to Leydig cell incubation resulted in androgen release. Increasing concentrations of T_3 causes a dose dependent

increases in androgen release indicating a biological response. Although it appears to be a very unusual effect of thyroid hormone, earlier report shows that thyroid hormone treatment may lead to a 1.5–2.5 fold increase in *in vitro* testicular testosterone synthesis in rat [28]. However what is more surprising in the present investigation is the inhibition of T_3 stimulated androgen release from the Leydig cell by cycloheximide. This suggests involvement of protein synthesis in the increased release of androgen. Role of thyroid hormone in protein synthesis is well known. It has been shown that T_3 specifically interacts with nuclear binding sites, triggering metabolic events which lead to the modulation of nuclear activity and results in stimulation of protein synthesis [4, 5, 31]. The above mentioned finding prompted us to observe T_3 effect on Leydig cell protein synthesis. Results clearly showed 70% increase in protein synthesis in Leydig cell by T_3 which is effectively inhibited by cycloheximide.

Our findings therefore show that a freshwater perch testicular Leydig cell nuclei contains a single species of high affinity and low capacity T_3 receptors which are saturable and analogue specific. These T_3 binding sites are possibly physiologically active as T_3 stimulates androgen release from Leydig cells. This stimulation appears to be not a direct effect of T_3 but mediated by a protein(s) induced by thyroid hormone. It is indeed a very interesting aspect and requires further validation in other teleosts.

ACKNOWLEDGMENTS

This work was supported by a grant from the Department of Sceince and Technology, Ministry of Science and Technology, New Delhi, India (SP/SO/CO3/88). The authors wish to express their sincere gratitude to Dr. John Y. L. Yu, Academia Sinica, Taipei, Taiwan for the generous gift of antiserum for androgen RIA.

REFERENCES

1 Anderson PH, Fukushima K, Schiller HS (1975) Radioimmunoassay of plasma testosterone with use of pollyethylene glycol to separate antibody bound and free hormones. Clin Chem 21: 708–714

2 Banerjee PP, Bhattacharya S, Nath P (1989) Purification and properties of pituitary gonadotropic hormone from Indian teleosts: Freshwater murrel (*Channa punctatus*) and carp (*Catla catla*). Gen Comp Endocrinol 73: 118–128

3 Benbrook D, Pfatl M (1987) A novel thyroid hormone receptor encoded by a C-DNA clone from a human testicular library. Science 238: 788–791

4 Bernal J, Coleoni AH, Degroot LJ (1978) Triiodothyronine stimulation of nuclear protein synthesis. Endocrinology 102: 452–459

5 Bernal J, Degroot LJ (1980) Mode of action of thyroid hormones. In "The thyroid gland" Ed by M De Vesseher, Raven Press, New York, pp 123–143

6 Chakraborti P, Bhattacharya S (1984) Plasma thyroxine levels in freshwater perch: Influence of season gonadotropins and gonadal hormones. Gen Comp Endocrinol 53: 179–186

7 Chakraborti P, Moitra G, Bhattacharya S (1986) Binding of thyroid hormone to isolated ovarian nuclei from a freshwater perch, *Anabas testudineus*. Gen Comp Endocrinol 62: 239–246

8 Chakraborti P, Rakshit DK, Bhattacharya S (1983) Influence of season gonadotropins and gonadal hormones on the thyroid activity of freshwater perch *Anabas testudineus* (Bloch). Can J Zool 61: 359–364

9 Darling DS, Dickhoff WW, Gorbman A (1982) Comparison of thyroid hormone binding to hepatic nuclei of the rat and a teleost (*Oncorhynchus kisutch*). Endocrinology 111: 1936–1942

10 Dettlaff TA, Davydova SI (1979) Differential sensitivity of cells of follicular epithelium and oocytes in the stellate sturgeon to unfavourable conditions and correlating influence of triiodothyronine. Gen Comp Endocrinol 39: 229–232

11 Dodd JM (1975) The hormones of sex and reproduction and their effects in fish and lower chordates twenty years on. Am Zool 15 (suppl 1): 127–171

12 Dufau ML, Mendelson CR, Catt KJ (1974) A highly sensitive *in vitro* bioassay for luteinizing hormone and chorionic gonadotropin: testosterone production by dispersed Leydig cells. J Clin Endocrinol Metab 39: 610–613

13 Eales JG (1979) Thyroid hormones in cyclostomes and fishes. In "Hormones and Evolution Vol 1" Ed by EJW Barington, pp 341–346

14 Fontaine YA (1975) Hormones in fishes. In "Biochemical and Biophysical perspective in marine biology Vol 2" Ed by DC Malins and JR Sargents, Academic Press, Orlando, Fla, pp 139–212

15 Gorbman A (1969) Thyroid function and its control in fishes. In "Fish Physiology Vol 2" Ed by WS Hoar and DJ Randal, Academic Press, New York, Vol 2: pp 241–274

16 Hurlburt ME (1977) Role of thyroid gland in ovarian maturation of the goldfish, *Carassius auratus*. L Can J Zool 55: 1906–1913

17 Ichikawa M, Mori T, Kawashima S, Ueda K, Shira-

hata S (1974) Histological changes in the thyroid and interrenal tissue of kokanee (*Oncorhynchus nerka*) during sexual maturation and spawning. J Fac Sci Univ Tokyo, 4: 175–182

18 Jannini AE, Olivieri M, Francavilla S, Gulino A, Ziparo E, D'armienoto M (1990) Ontogenesis of the nuclear 3,5,3′-triiodothyronine receptor in the rat testis. Endocrinology 126: 2551–2526

19 Lawson GM, Tsai MJ, Tsai SY, Minghetti PP, McClure ME, O'Malley BW (1982) In "Laboratory Methods Manual for Hormone action and Molecular Endocrinology". Ed by H Ballin and BW O'Malley, 6th Ed Houston Biological Assn Inc, pp 5–7

20 Leatham J (1972) Role of Thyroid. In "Reproductive Biology" Ed by H Ballin and S Glasser, Experta Medica, Amsterdam, pp 857–876

21 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193: 265–275

22 Maitra G, Bhattacharya S (1989) Seasonal changes of Triiodothyronine binding to piscine ovarian nuclei. Zool Sci 6: 771–775

23 Matty AJ (1960) Thyroid cycles in fish. Symp Zool Soc London, 2: 1–15

24 Oppenheimer JH (1979) Thyroid hormone action at the cellular level. Science 203: 971–979

25 Oppenheimer JH, Schwartz HL, Surks MI (1974) Tissue differences in the concentration of triiodothyronine nuclear binding sites in the rat: liver, kidney, pituitary, heart, brain, spleen and testis. Endocrinology 95: 897–903.

26 Sage M (1973) The evolution of thyroidal function in fishes. Am Zool 13: 899–905

27 Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B (1986) The C-erb-A protein is a high-affinity receptor for thyroid hormone. Nature 324: 635–640

28 Schneider G, Kopach K, Ohanian H, Bonnefond V, Mittler JC, Ertel NH (1979) The hypothalamic-pituitary-gonadal axis during hyperthyroidism in the rat. Endocrinology 105: 674–679

29 Sen S, Bhattacharya S (1981) Role of thyroid and gonadotropin on the mobilization of cholesterol in a teleost, *Anabas testudineus* (Bloch). Indian J Exp Biol 19: 408–412

30 Sen S, Bhattacharya S (1982) Hormonal influence of perch ovarian 17β-hydroxysteroid dehydrogenase activity in *in vitro* system. Indian J Exp Biol 20: 664–667

31 Tata JR, Widnell CC (1966) Ribonucleic acid synthesis during early action of thyroid hormones. Biochem J 98: 604–620

32 Van Damme MP, Robertson DM, Diczfalusy E (1974) An improved *in vitro* bioassay method for measuring luteinizing hormone (LH) activity using mouse Leydig cell preparations. Acta Endrocrinol 77: 655–671

33 Vander Kraak JG, Eales JG (1980) Saturable 3,5,3′-triiodothyronine binding sites in liver nuclei of rainbow trout (*Salmo gairdneri*). Gen Comp Endocrinol 42: 437–448

34 Weinberger C, Thompson CC, Ong SE, Lebo R, Gruol JD, Evans MR (1986) The C-erb-A gene encodes a thyroid hormone receptor. Nature 324: 641–646

35 Wiebe PJ (1978) Steroidogenesis in rat Leydig cells: Effect of gonadotropin on the activity of 5-Ane and 5-Ene 3α- and 3β-hydroxysteroid dehydrogenases during sextual maturation. Endocrinology 102: 765–784

36 Yu JYL, Chang TY, Hsu HK, Liao CF, Wan WCM (1981) Androgen/testosterone synthesis by the dissociated testicular cells from mice of different ages in response to rat LH stimulation *in vitro*. Bull Inst Zool Acad Sin 20: 57–65

37 Yu JYL, Wang LM (1987) Comparative effect of diverse vertebrate gonadotropins on androgen formation *in vitro* from testes of roosters and mice. Biol Reprod 36: 816–824